# The Influence of a Matrix Metalloproteinase on the Remineralization of Artificially Demineralized Dentin

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**Purpose:** This investigation aimed at determining whether a collagenolytic matrix metalloproteinase (MMP-8) present in the oral fluids might attack demineralized dentin and thereby affect the potential for remineralization.

**Materials and Methods:** Dentin specimens from freshly extracted third molars were demineralized for 14 days *in vitro* and then remineralized for 7 days in the presence or absence of activated MMP-8. Following treatment the mineral level of the specimens was assessed from the outer dentinal surface using Electron Probe Micro Analysis (EPMA).

**Results:** The findings suggested that the addition of activated MMP-8 to the remineralizing solution led to reduced remineralization in the surface of the dentin. There appeared to be no effect on the remineralization from any dentin bound MMP activity.

**Conclusion:** It appears that MMP-8 may negatively influence the remineralization of demineralized dentin.

**Key words:** dentin, matrix metalloproteinase, remineralization

*Oral Health Prev Dent 2003; 1: 267–272. Submitted for publication: 07.05.03; accepted for publication: 07.10.03.*

It has previously been shown that certain matrix metalloproteinases (MMPs) present in saliva and gingival crevicular fluid are active during carious breakdown of the dentin matrix (Tjäderhane et al, 1998). Furthermore, inhibition of MMPs is reported to reduce the progression of caries in rats (Sulkala et al, 2001). Like some other MMPs, MMP-8, which is inactive when synthesized, is activated when pH is lowered to pH 4.5 and then rises to closer to neutral (Tjäderhane et al, 1998). These conditions mimic those found during caries progression and in erosive processes. There are data showing that dentin, demineralized *in vitro* by demineralizing solutions, can be remineralized and even hypermineralized, under favorable *in vitro* conditions (Arends et al, 1997; Heilman et al, 1997; Clarkson et al, 1991, 1998). However, the artificially demineralized dentin probably does not adequately simulate the conditions encountered in caries-affected dentin, where degradation of the organic matrix is an additional characteristic. Dentin collagen itself does not seem to interfere with the remineralization of dentinal lesions (Klont and ten Cate, 1991; Featherstone, 1994; ten Cate, 2001). However, it may constitute a necessary support for the maintenance of the original level of the surface (Kawasaki and Featherstone, 1997).

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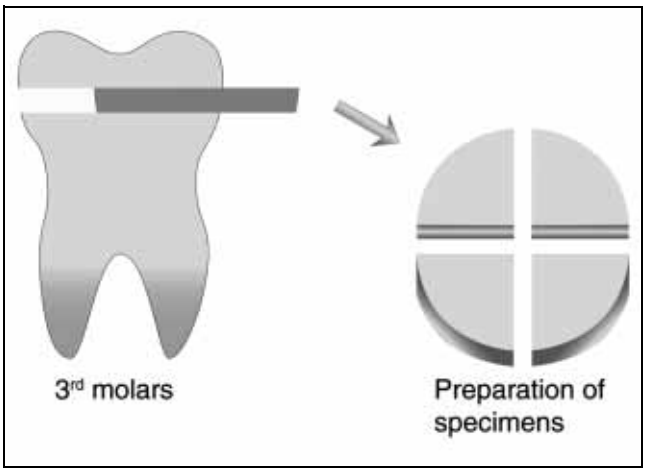


**Fig 1** Preparation of dentin specimens.

The degradation of exposed collagen in dental caries and erosive processes may be caused by the combined effects of different MMPs, since a telopeptidase activity may be necessary to remove the crosslink containing terminal peptides of the collagen fibrils as the primary step prior to degradation of the fibrils (Eeckhout et al, 1988; Tjäderhane et al, 1998). Of the three MMPs (MMP-2, MMP-8, and MMP-9) found in saliva and carious dentin, MMP-9, which is gelatinolytic, has been shown to have such activity (Tjäderhane et al, 1998).

The present investigation aimed at studying whether the presence of a simplified oral collagenolytic system might also influence the remineralization of the surface layer of artificially demineralized dentin. The hypothesis tested was that such collagenolytic activity on partially denatured and mineral depleted collagen, following demineralization, will reduce the remineralization of the outer dentin layer, thereby contributing to loss of tissue. In our *in vitro* model the collagenolytic MMP-8, present in carious lesions and active in hydrolyzing collagen type 1 (Palosaari et al, 2000), was therefore introduced to remineralizing systems.

## MATERIALS AND METHODS

### *Solutions*

Demineralizing solution:

Acetic acid, 0.05 moles/L; $CaHPO_4$, 2.2 mmoles/L; pH 4.3. Thymol at 0.03% was added to the stock solution to prevent microbial growth.

Remineralizing solution (Iijima et al, 1993):

HEPES buffer, 20 mmoles/L; Calcium, 1.5 mmoles/L as $CaCl_2$; phosphate, 0.9 mmoles/L as $K_2HPO_4$; fluoride, 10 ppm as NaF; pH 7.0.

Matrix metalloproteinase:

Matrix metalloproteinase-8 (MMP-8) was purchased from Chemicon Int., Temecula, Ca., USA. According to Chemicon's recommendations it was activated with Trypsin (Sigma Chem Co, USA) (5 µg Trypsin/ml, pH 7.6, 37 °C) for 1 h, and the activation process was then stopped with Soy Bean Inhibitor (Sigma Chem Co, USA.) (1 µg Inhibitor/1.5 µg Trypsin).

Proteinase inhibitor:

TIMP-2 was recommended by and obtained from Chemicon Int., Temecula, Ca., USA.

### *Preparation of Specimens and Experimental Conditions*

Slices of dentin (3–5 mm thick) were cut horizontally through the crowns of 36 freshly extracted, caries free 3rd molars (from patients aged 20–35 yr) that had been kept in Ringer's solution at 4°C for no longer than 2 days. The slices (Fig 1) were divided vertically into 4 equal quadrants.

One quadrant from each tooth (Quadrant no. 1) was maintained untreated at 4°C in 100% humidity as a control of the natural mineral level of the dentin in the relevant area. The other 3 quadrants from each tooth were covered with nail varnish on all sides except for the occlusal dentin surface (area approximately 5 $mm^2$), and were then immersed in 25 ml of the demineralizing solution for 14 days at 37°C and pH 4.3 (Ngo et al, 1997).

One of these quadrants (Quadrant no. 2) from each tooth received no further treatment and was maintained in 100% humidity at 4°C as a control of the level of demineralization.

Quadrant no. 3 was exposed for 7 days at 37°C to 25 ml of the remineralizing solution to establish the 'normal' remineralization pattern of the dentin in the relevant area.

Quadrant no. 4 was exposed to one of the following routines:

a) Kept in 25 ml of a neutral buffer (0.1 M HEPES buffer at pH 7.0) for 2 days followed by storage in 25 ml of the remineralizing solution for 7 days in an attempt to reveal the possible presence of dentin-bound MMP activity (Palosaari et al, 2000). In total: 12 quadrants.

b) Stored in 25 ml of the remineralizing solution to which TIMP-2 (0.30 μg) had been added to inhibit any dentine-bound MMP activity (Control 1). In total: 8 quadrants.
c) Stored in 25 ml of the remineralizing solution to which 0.55 μg of activated MMP-8 had been added. In total: 8 quadrants.
d) Stored in 25 ml of the remineralizing solution to which activated MMP-8 (0.55 μg) and TIMP-2 (0.30 μg) had been added (Control 2). In total: 8 quadrants.

The nail varnish was then removed and all specimens were fixed by storage overnight in 2.5% glutaraldehyde in 0.1% sodium cacodylate buffer at pH 7.4 at 4 °C. Subsequently the specimens were rinsed three times for one hour each time in 0.2 M sodium cacodylate buffer at pH 7.4 followed by rinsing in distilled water. They were dehydrated in ethanol at concentrations from 25% to 100%, then dried in hexamethyldisilisane for 10 minutes before air drying for 3–5 minutes. Subsequently the specimens were embedded in epoxy resin LC 191 (Batch 682192) and HY 956 (Batch 681406) ensuring that a surface perpendicular to the experimental occlusal surface was level with the flat surface of the block produced. This surface was then ground and polished with sand paper disks, grit 500 and 1000, followed by aluminum disks from 15 μm down to 1 μm.

### *Assessment of Mineralization Level*

The specimens were line scanned by Electron Probe Microanalysis (EPMA) (Chu et al, 1989; Ngo et al, 1997) to measure the concentration (and relative concentration) of calcium, phosphorus, and fluorine lineally from the experimental surface and inward, thus assessing the mineralization level following the de- and remineralization procedures. The concentration of calcium and phosphorus relates allowably with the general mineralization level of the dental hard tissues (Ngo et al, 1997; Arends et al, 1997). Sodium was also registered but had no relevance in this context. Wilberforce fluorapatite was used as a standard and the PAP-modified version of the ZAF software package was used for correction. The specimens were first examined in a stereomicroscope at 5–10 x to examine the thickness and evenness of the de- and remineralized zones in order to find areas without flaws and with even and distinct boundary lines for the line scanning. Spot analysis using an electron probe analyzer (Cameca SX 51) was performed at 2 μm intervals along a transverse line perpendicular to the outer surface. The excitation voltage was kept at 15kV and the current at 20nA. The diameter of the electron beam was 2 μm, and counting time was 10 sec. at each point. A set of 7 lines was performed for each sample. The raw data were treated using ZAF correction to produce curves indicating the de- or remineralization pattern from the experimental surface and inward (Ngo et al, 1997).

## RESULTS AND DISCUSSION

The Electron Probe Microanalysis (EPMA) method has been described and discussed elsewhere (Chu et al, 1989; Ngo et al, 1997). Briefly, it is based on the emission of the characteristic x-ray radiation of elements within a small volume of the sample excited by the impact of a focused beam of electrons of appropriate energy, giving minimum detection limits typically less than 1% (Chu et al, 1989; Ngo et al, 1997). The use of de- and remineralizing solutions as outlined in the present study represents a simple and accepted method of studying de- and remineralization processes *in vitro* (Featherstone, 1994; Kawasaki and Featherstone, 1997; Iijima et al, 1993; Ngo et al, 1997).

A distinct zone of demineralization was found in the dentinal surface following exposure to the demineralizing solution for 14 days, as typically demonstrated in Fig 2A. This zone exhibited some variation in depth among the teeth, which was to be expected when taking into consideration the range of compositional differences normally found in the dentin (Marshall et al, 1997).

Following exposure to the remineralizing solution for 7 days the demineralized zone in the surface layer generally showed a remineralization up to the initial mineral level, as demonstrated in Fig 2B. This is in agreement with a previous investigation (Heilman et al, 1997); however, in contrast to the hypermineralization found by Iijima et al (1993). As expected, the remineralized zones exhibited some variations when comparing specimens from different teeth. Out of the 36 demineralized specimens (from 36 teeth), 2 exhibited no traceable remineralization, and 2 others obtained values representing about one third of the initial mineral level. Wefel et al (1995) and Clarkson et al (1998) have shown

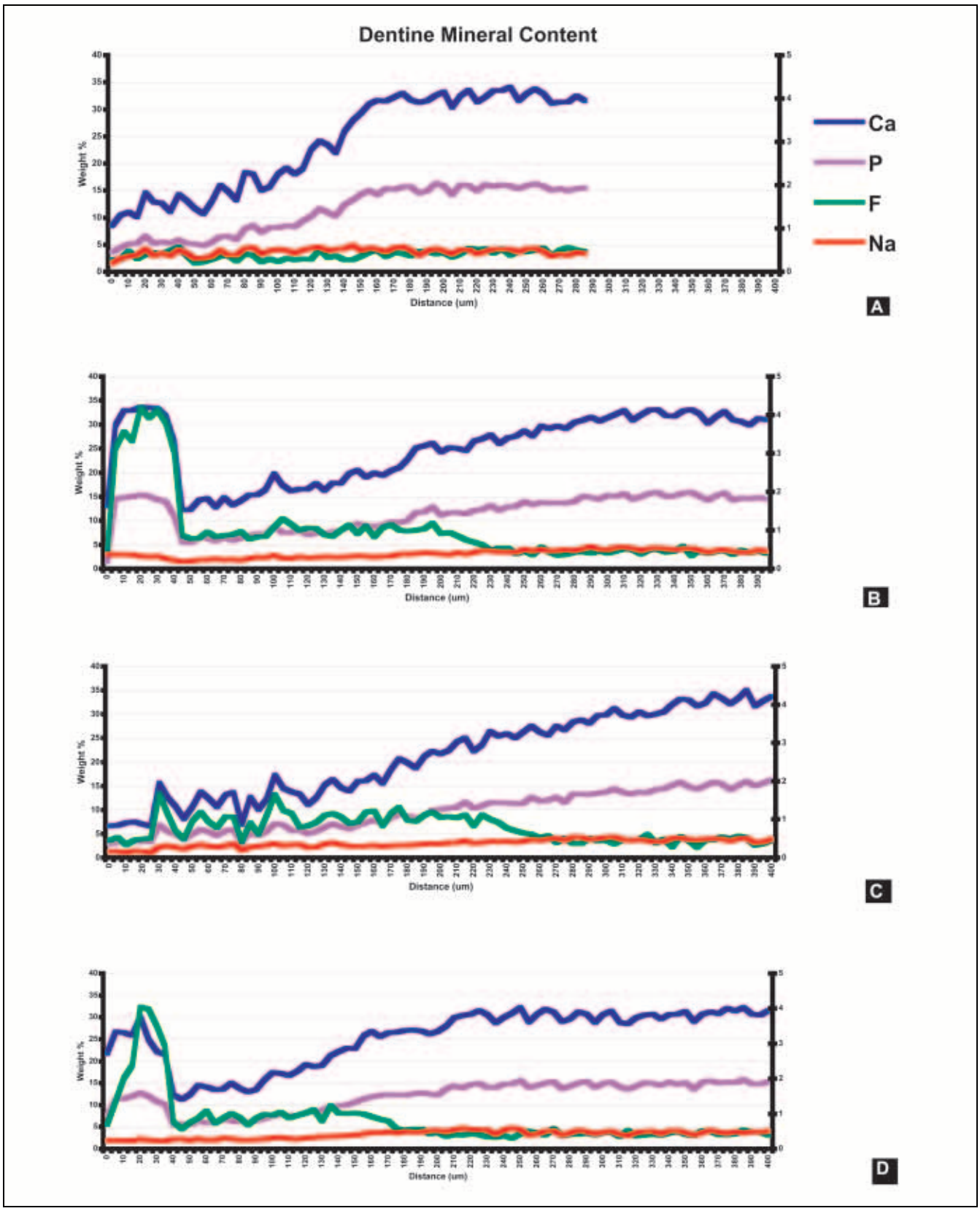


**Fig 2** Mineral content in the surface of dentin in a typical tooth sample after: **(A)** Demineralization **(B)** Demineralization followed by remineralization **(C)** Demineralization followed by remineralization in the presence of MMP-8 **(D)** Demineralization followed by remineralization in the presence of MMP-8 and TIMP-2.

that the organic matrix remaining after complete demineralization appears to be unable to act as a suitable substrate for remineralization, because remineralization can occur only in the presence of some remaining mineral. Thus, for some reason, extensive demineralization may have taken place in the 4 particular specimens and may possibly account for the low or lacking remineralization observed. Due to the interdental variation great care was taken to use neighboring areas of dentin from the same tooth when comparing the effect of different treatments, as illustrated in Fig 2.

To study whether inactive forms of MMPs might be present in dentin (Martin-De Las Heras et al, 2000; Palosaari et al, 2000), 12 specimens, following demineralization at pH 4.3, were placed in a calcium- and phosphate-free buffer (HEPES buffer) at neutral pH for 2 days in an effort to activate any enzyme present, before being transferred to the remineralizing solution. No enzymatic effect could, however, be demonstrated by the EPMA analyses.

When activated MMP-8 was incorporated in the remineralizing solution, areas of thinning and/or indistinctness of the demineralized zone could be seen microscopically in 5 out of the 8 specimens. The EPMA assessment showed a remineralization in the surface layer in those specimens that was only 30–35% of that found without enzyme (Fig 2; C compared to B), indicating an enzymatic effect. The remineralization pattern in the 5 specimens exhibited a uniform tendency. In the remaining 3 specimens an 80–90% remineralization was demonstrated, compared to that obtained without enzyme, indicating a much lower (or no) enzymatic effect.

When the enzyme inhibitor TIMP-2 was present the remineralization proceeded as in samples without enzyme (Fig 2; D compared to B), except in two out of the 8 specimens. The latter might possibly be due to extensive demineralization in those specimens.

It is suggested that MMP-8 alone, like other proteases (Beltz et al, 1999; Linde and Goldberg, 1993; Kawasaki and Featherstone, 1997; Walters and Eyre, 1983) may not be capable of breaking down collagen protected by mineral, or fibrils of collagen type 1 without some preceding solubilization of the fibrillar collagen (Eeckhout et al, 1988; Tjäderhane et al, 1998). In the present experiment the varying effect of MMP-8 might therefore be due to interdental variations in mineral levels, 'contamination' with telopeptidase activity (from traces of MMP-9), and/or a varying solubility state of the fibrillar collagen caused by acid denaturation. Also, any activity of dentin sialoproteins and dentin phosphoproteins associated with the collagen and supposed to participate in dentin mineralization might be affected by an enzymatic attack on the collagen (Liu et al, 2002; Ritchie et al, 2002).

Thus, within the limits of the present study it may be postulated that MMP-8 activity during remineralization cycles may disturb the remineralization process and thereby contribute to a reduction of the dentin surface.

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